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Review

Factors That Influence Primary Cilium Length

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Almost all mammalian cells carry one primary cilium that functions as a biosensor for chemical and mechanical stimuli. Genetic damages that compromise cilia formation or function cause a spectrum of disorders referred to as ciliapathies. Recent studies have demonstrated that some pharmacological agents and extracellular environmental changes can alter primary cilium length. Renal injury is a well-known example of an environmental insult that triggers cilia length modification. Lithium treatment causes primary cilia to extend in several cell types including neuronal cells; this phenomenon is likely independent of glycogen synthase kinase-3β inhibition. In renal epithelial cell lines, deflection of the primary cilia by fluid shear shortens them by reducing the intracellular cyclic AMP level, leading to a subsequent decrease in mechanosensitivity to fluid shear. Primary cilium length is also influenced by the dynamics of actin filaments and microtubules through the levels of soluble tubulin in the cytosol available for primary cilia extension. Thus, mammalian cells can adapt to the extracellular environment by modulating the primary cilium length, and this feedback system utilizing primary cilia might exist throughout the mammalian body. Further investigation is required concerning the precise molecular mechanisms underlying the control of primary cilium length in response to environmental factors.

Key words: primary cilium length, lithium, cyclic AMP, soluble tubulin, intraflagellar transport

It is well recognized that multiple motile cilia on epithelial cells of the respiratory and reproductive tracts and the ependyma generate fluid flow, while almost all cells throughout the mammalian body are known to have an immotile primary cilium, a cell surface organelle that contains a membrane-bound microtubule axoneme in the center. The primary cilium singly extends like an antenna into the extracellular environment surrounding the cell and transduces sensory stimuli to the cell body (Fig. 1A) [1, 2]. A fine cilia structure with a high surface-volume ratio is thought to allow efficient signal amplification.

Approximately 200 genes that are absent in the genomes of nonciliated eukaryotes are conserved in ciliated organisms [3]. Damages to genes needed for the formation and function of primary cilia result in a spectrum of disorders with different manifestations such as Bardet-Biedl syndrome (BBS), Joubert syndrome, Meckel-Gruber syndrome and nephronophthisis; these disorders are now classified as ciliopathies [4, 5]. Common clinical features of ciliopathies include renal abnormality, retinal degeneration, polydactyly, cognitive dysfunction and obesity.

Intraflagellar transport (IFT) is a highly conserved system and crucial for the formation and maintenance

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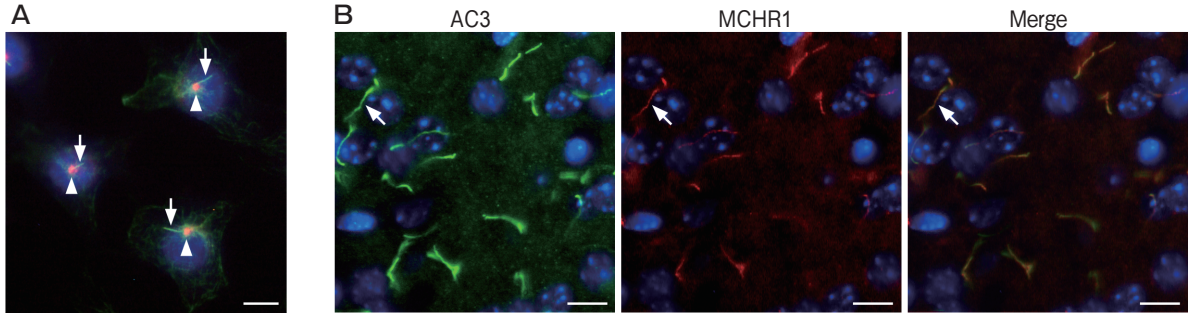

Fig. 1 Almost all mammalian cells, including neuronal cells, have a single nonmotile cilium, known as the primary cilium, that functions as a biosensor. **A**, Mouse fibroblast NIH3T3 cells carry one primary cilium protruding from the basal bodies. Primary cilia (arrows) and basal bodies (arrowheads) are labeled with antibodies to acetylated tubulin and pericentrin, respectively; **B**, Adenylyl cyclase (AC) 3 and melanin-concentrating hormone receptor 1 (MCHR1) co-localize to the primary cilia (arrows) of neurons in the mouse striatum. Nuclei are stained with Hoechst (blue). Scale bar, 10 μm.

of flagella and cilia [5, 6]. IFT particles, protein complexes composed of several subunits, deliver cargo along microtubules by means of motor proteins between the cell body and the tip of the flagellum and cilium. *Tg737*^{orpk} mice that carry a hypomorphic mutation in the gene encoding the IFT88 subunit exhibit renal cysts, hydrocephalus and ductal abnormalities in the liver and the pancreas and die shortly after birth [7–11]. *Tg737*^{orpk} mice have underdeveloped primary cilia in the renal, biliary and pancreatic epithelia as well as malformed ependymal cilia [8–11], indicating that disruption of the IFT machinery results in systemic and lethal abnormalities. On the other hand, selective structural defects in the chemosensory cilia of olfactory neurons have been found in mice due to the disruption of causative genes for BBS [12, 13] as well as pericentrin [14]. The reasons that the disturbance of a particular cilia-related gene affects some particular subset of ciliary functional or structural features while leaving others intact are uncertain [15].

Besides genetic damage to ciliary genes, increasing evidence suggests that chemical and mechanical stimuli can affect the architecture of cilia [16–24]. In this article, we review recent studies on the environmental and pharmacological factors that affect the length of primary cilia in mammalian cells.

Renal Injury Induces Elongation of Primary Cilia

A well-known example of environmental insults that trigger the alteration of cilia length is renal injury. Primary cilia on renal tubular epithelial cells function as flow sensors that are required for normal epithelial proliferation and differentiation in the kidney [25, 26]. Defects in renal primary cilia cause dysregulated proliferation and differentiation, resulting in polycystic kidney disease [26–28]. It has been shown that the lengths of renal primary cilia increase in murine-injured kidneys [16–18] and in human renal transplants that suffer acute tubular necrosis [18]. Verghese *et al.* recently analyzed the factors that contribute to the elongation of renal primary cilia using Madin Darby canine kidney (MDCK) cells that carry primary cilia [19]. MDCK cells treated with cobalt chloride ($CoCl_2$) that stabilizes the hypoxia-inducible factor alpha (HIF-1α) transcription factor, simulating injury-associated hypoxia in the kidney, have revealed much longer cilia compared to control cells. It has been proposed that the elongation of renal primary cilia following renal injury would increase their sensory capacity, which might be crucial to epithelial differentiation during renal repair.

Lithium Treatment Elongates Primary Cilia

The biflagellate algae *Chlamydomonas reinhardtii* is a good model organism for the study of flagella, projections which are analogous to vertebrate cilia. It has been known that the treatment of algae with LiCl induces elongation of the flagella in algae [29–31]. Lithium (Li) is a classical mood-stabilizer used for both the prophylactic treatment of bipolar affective

disorder and the acute treatment of mania. An inhibition of glycogen synthase kinase-3β (GSK3β) is thought to underlie the mood-stabilizing effects of Li, while GSK3β-independent mechanisms have also been suggested [32, 33]. It is noteworthy that LiCl treatment has also been shown to inhibit the kinase activity of glycogen synthase kinase-3 in algae [29].

In the rodent central nervous system (CNS), each neuron seems to be equipped with a single primary cilium for nearly all regions [34–39], although the biological roles played by the primary cilia in the CNS remain uncertain. In the murine CNS, adenylyl cyclase (AC) 3 [40, 41] and two G protein-coupled receptors (GPCR) for neuropeptides, namely melanin-concentrating hormone receptor 1 (MCHR1) [42] and somatostatin receptor type 3 [43–45], have been found to be localized on the neuronal primary cilia (Fig. 1B). AC catalyzes the conversion of ATP to cyclic AMP (cAMP). AC3 and chemosensory GPCR in the specialized cilia of the olfactory receptor neurons are known to play key roles in odorant signal transduction [46]. This suggests that a G protein/cAMP signaling cascade also exists in the CNS neuronal primary cilia, transducing chemical stimuli in the extracellular space to the cell body. The melanin-concentrating hormone system is thought to regulate energy homeostasis and emotional processing [47, 48], and to modulate dopaminergic function [49–52].

We have shown that Li treatment elongates primary cilia in the mouse brain and in cultured cells [20]. The effects of chronic Li administration on primary cilia have been investigated in the mouse striatum, which receives major dopaminergic projections from the midbrain [51] and abundantly expresses MCHR1 on the primary cilia of neuronal cells [42]. Brain sections from mice fed with Li_2CO_3-containing food pellets for 16 days have been subjected to immunofluorescence study; this dietary treatment with Li has resulted in a serum Li level of 2.1 ± 0.2 mM, which is above the maximum therapeutic serum level in humans (0.5–1.5 mM). Primary cilia expressing both AC3 and MCHR1 have been significantly longer (approximately 1.3-fold) in Li-fed mice than in control animals in the dorsal striatum and nucleus accumbens. Mouse fibroblast NIH3T3 cells have exhibited a significant increase in the length of primary cilia after treatment with 5–50 mM LiCl for 24 h in a dose-dependent manner as compared to untreated cells, while treatment with 50 mM NaCl has also led to a significant increase in the length of primary cilia. Treatment with 50 mM LiCl has evoked gradual elongation of NIH3T3 primary cilia up to 16 h, with the primary cilia becoming twice as long as the untreated cilia. A 24 h-treatment with 25 mM or 50 mM KCl has also induced a significant increase in the length of primary cilia in NIH3T3 cells, whereas the cilia length of $MgCl_2$- or $CaCl_2$-treated cells has been comparable to that of untreated cells. The order of the mean cilia lengths of NIH3T3 cells treated with 50 mM monovalent cation chlorides for 24 h has been LiCl > KCl > NaCl. Interestingly, treatment with a synthetic GSK3β-specific inhibitor, SB216763 or TDZD-8, has shown no significant effect on cilia lengths in NIH3T3 cells. Treatment of cultured primary cells prepared from the striata of rat embryos with 2.5 mM LiCl for 40 h has caused elongation of the AC3-expressing primary cilia of neurons, while 2.5 mM NaCl-treatment has had no effect. These findings suggest the participation of Li-sensitive targets other than GSK3β [32, 33] in the elongation of the primary cilia and further raise the possibility of a contribution of certain actions common to monovalent cations to the Li effect on the length of primary cilia in the mouse brain and in cultured cells.

Primary cilia elongation caused by lithium treatment has also been reported by Ou *et al.* [21] in a study in which fibroblast-like synoviocytes (FLS) were mainly analyzed. Treatment with 5–100 mM LiCl has led to a significant increase in the primary cilium length of FLS cells in a dose-dependent manner. FLS cells treated with 100 mM LiCl for 12 h have grown primary cilia that were 3 times longer than those of untreated cells, and have exhibited their longest cilia at the 21 h time point after treatment. In contrast to our observation in NIH3T3 cells, the cilia length of FLS cells has not changed after treatment with NaCl, suggesting that cilium elongation is specific to Li. Treatment with GSK3β inhibitors has failed to alter the length of primary cilia, while inhibition of AC3, another Li-sensitive target, by adenosine 3' monophosphate or dideoxyadenosine triphosphate has led to primary cilia elongation in FLS cells. AC3 expression has been detected in the primary cilia not only of FLS cells but also of human foreskin fibroblasts, NIH3T3 cells, rat PC12 cells and human astrocytes. Further, Li-induced elongation of primary cilia has

been observed in all of these cells. It is notable that the primary cilia of neuron-like PC12 cells and human astrocytes have been elongated after treatment with therapeutic concentrations of Li (1–2 mM). These results suggest that Li treatment increases primary cilium length, at least in part, via the inhibition of AC3 and a consequent decrease in the cAMP level.

Primary Cilia Deflection Shortens Primary Cilia via Regulation of the cAMP Level

Besschetnova *et al.* have made contradictory findings with regard to the cAMP effect on the length control of primary cilia [22]. They have demonstrated that the activation of AC by treatment with forskolin or the inhibition of intracellular Ca^{2+} entry by treatment with Gd^{3+} elongates primary cilia in inner medullary collecting duct (IMCD) cells, embryonic kidney epithelial (MEK) cells and primary bone mesenchymal cells. Further, they have provided evidence that crosstalk between a decrease in the intracellular Ca^{2+} and an increase in the cAMP level with subsequent protein kinase A activation mediates the elongation of primary cilia (Fig. 2). Interestingly, visualization of the IFT88 subunit fused to enhanced yellow fluorescent protein in live IMCD cells has revealed that treatment with Gd^{3+} or forskolin increases anterograde IFT velocity with no effect on retrograde velocity. Furthermore, deflection of the primary cilia by fluid shear has been shown to decrease the intracellular cAMP level in IMCD and MEK cells, thereby leading to the shortening of primary cilia and a subsequent decrease in mechanosensitivity to fluid shear. This negative feedback response has been disrupted in IMCD and MEK cells knocked down for polycystin-2 or mutated for polycystin-1, respectively, which are products of the genes for autosomal-dominant polycystic kidney disease [26–28].

Cytoskeletal Dynamics Regulate Primary Cilium Length

Kim *et al.* have identified the human genes involved in the assembly and disassembly of primary cilia by a high-throughput functional genomic screen using RNA interference [23]. *ACTR3*, one of the identified cil-

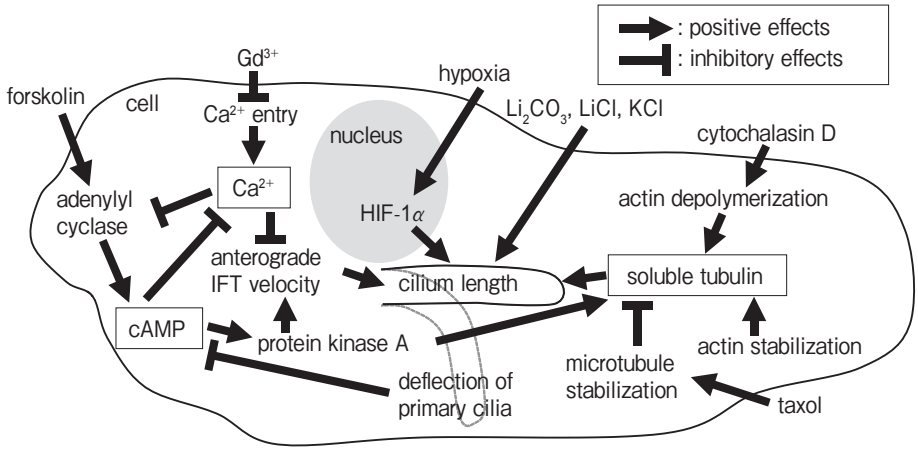

Fig. 2 Proposed pathways by which environmental and pharmacological factors influence primary cilium length. Treatment with Gd^{3+} or forskolin evokes the elongation of primary cilia via crosstalk between a decrease in the intracellular Ca^{2+} and an increase in the cyclic AMP (cAMP) level with subsequent protein kinase A activation [22]. The deflection of primary cilia downregulates the intracellular cAMP level, resulting in primary cilia shortening and a subsequent decrease in the mechanosensitivity of primary cilia [22]. Increased velocity of anterograde IFT is thought to underlie the cAMP/Ca^{2+}-mediated elongation of primary cilia [22]. Treatment with cytochalasin D or taxol affects the dynamics of actin filaments and microtubules, respectively, leading to cilia length alteration through modulating the levels of soluble tubulin available for primary cilia extension [24]. Forskolin-induced elongation of primary cilia is attenuated by treatment with taxol, where the effects on cilia length correlate with the levels of soluble tubulin [24]. The mechanism by which treatment with Li^+ [20, 21] or K^+ [20] elongates primary cilia is uncertain but might be related to certain actions common to monovalent cations. Among the consequences of renal injury, hypoxia is likely to contribute to the elongation of renal primary cilia via hypoxia-inducible factor alpha (HIF-1α) [19].

iogenesis modulators, has been known to encode an actin-related protein required for the nucleation of actin polymerization at filament branches [53]. *ACTR3* knockdown has led to the elongation of primary cilia, suggesting an inhibitory role of actin polymerization in the formation of cilia. Treatment with cytochalasin D, an inhibitor of actin polymerization, has facilitated ciliogenesis in telomerase-immortalized human retinal pigmented epithelial (htRPE) cells, confirming the relation between actin dynamics and primary cilia assembly. Further, Kim *et al.* have also demonstrated that the inhibition of actin polymerization facilitates ciliogenesis by stabilizing the pericentrosomal preciliary compartment, a compact vesiculotubular structure containing proteins to be incorporated into the cilia.

Sharma *et al.* have demonstrated that not only actin depolymerization by treatment with cytochalasin D but also actin stabilization by treatment with jasplakinolide leads to the elongation of primary cilia in multiple cell types including htRPE and IMCD cells [24]. Moreover, treatment with taxol that stabilizes microtubules and decreases the cytosolic free tubulin has inhibited the primary cilia elongation caused by either actin depolymerizing or stabilizing agents. These effects on primary cilium length have correlated with the levels of soluble tubulin in the cytosol available for primary cilia elongation. They have also demonstrated that forskolin-induced elongation of primary cilia is attenuated by treatment with taxol, where the effects on cilia length correlate with the levels of soluble tubulin. These findings suggest that modification of microtubule and actin cytoskeletons influences primary cilium length via soluble tubulin levels in the cytosol, and they further present an alternative explanation for cAMP-mediated regulation of cilia length.

IFT and Primary Cilium Length

It is widely recognized that a balance between the assembly and disassembly of microtubules in axonemes regulates flagella and cilia length. IFT particles transport precursors to the distal tips of axonemal microtubules, where continuous turnover occurs [54]. Thus, equilibration at an increased length can arise from either an increase in the assembly rate or a decrease in the disassembly rate. If a simple steady-state model for the length control of algae flagella [54] underlies cilia length regulation in mammalian cells, potential control points for length modulation by environmental factors will contain the velocity of each IFT particle, the number of active IFT particles and a length-independent disassembly rate. It is noteworthy in this context that stimuli that elongate primary cilia increase anterograde IFT velocity without changing retrograde velocity in live IMCD cells [22].

Conclusions

Primary cilium length would parallel the sensitivity of the organelle to chemical stimuli if the receptor concentrations on the primary cilia are constant, as well as to mechanical stimuli. Therefore, cilium length modulation would allow the adaptation to environmental changes such as altered concentrations of substances, excessive fluid flow and oxygen deficiency. It is possible that this feedback system depending on primary cilium length plays an important role throughout the mammalian body. The interplay among the levels of Ca^{2+}, cAMP and soluble tubulin seems to be essential for the regulation of cilia length (Fig. 2). Further investigation is needed to elucidate the precise molecular mechanisms controlling the length of primary cilia in response to environmental factors.

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